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SURVEY AND SUMMARY

The use of diaminopurine to investigate structural properties of nucleic acids and molecular recognition between ligands and DNA

Christian Bailly* and Michael J. Waring[1]

INSERM U-124 et Laboratoire de Pharmacologie Antitumorale du Centre Oscar Lambret, IRCL, Place de Verdun, 59045 Lille, France and [1]Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QJ, UK

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ABSTRACT

2,6-Diaminopurine (DAP) is an analogue of adenine which can be converted to nucleotides that serve as substrates for incorporation into nucleic acids by polymerases in place of (d)AMP. It pairs with thymidine (or uracil), engaging in three hydrogen bonds of the Watson–Crick type. The result of DAP incorporation is to add considerable stability to the double helix and to impart other structural features, such as an altered groove width and disruption of the normal spine of hydration. DNA containing DAP may or may not be recognized by restriction endonucleases; RNA containing DAP may not engage in normal splicing. The DAP·T pair affects the local flexibility of DNA and impedes the interaction with helix bending proteins. By providing a non-canonical hydrogen bond donor in the minor groove and/or blocking access to the floor of that groove it strongly affects interactions with small molecules such as antibiotics and anticancer drugs. Examples which illustrate altered recognition of nucleotide sequences in DAP-containing DNA are presented: changed sites of cutting by bleomycin, photocleavage by uranyl nitrate and footprinting with mithramycin. Using DNA in which both A→DAP and G→Inosine substitutions have been made it is possible to assess precisely the role of the purine 2-amino group in ligand–DNA recognition.

INTRODUCTION

It was exactly half a century ago when the first modified nucleobase, 5-methylcytosine (5-MeC), was discovered (1). Nowadays, it is well established that this unusual but naturally occurring base participates in the control of gene expression in higher organisms (2). Over the last 50 years many other modified bases have been discovered in DNA. One of the most interesting is 2-aminoadenine (abbreviated to DAP or D for 2,6-diaminopurine), which is used in place of adenine by the cyanophage S-2L (3,4), whose very existence shows that DAP in DNA is compatible with normal DNA function (5,6).

The DAP·T base pair possesses an extra hydrogen bond compared with A·T because of the additional -NH_2 pointing toward the minor groove of DNA (Fig. 1). DAP is a common tool in nucleic acid chemistry which can be used to study molecular recognition between DNA or RNA and ligands, both small and large. In this paper, various applications of DAP are briefly presented. In particular, the emphasis is on how this base can be extremely useful in investigations on the structure of nucleic acids as well as sequence-specific interactions between DNA and small molecules or proteins.

CHEMICAL AND ENZYMATIC SYNTHESIS OF DIAMINOPURINE-CONTAINING DNA

In most cases, the DAP base is introduced chemically into DNA sequences using conventional phosphoramidite chemistry. Synthesis of the DAP nucleoside phosphoramidite has been described (7). Alternatively, DAP can be incorporated into DNA by enzymatic methods via the use of DAP triphosphate and polymerases. The triphosphate of 2-aminoadenosine acts as a true analogue of ATP in transcription (8). 2,6-Diaminopurine-2′-deoxyribonucleoside is commercially available and methods have been described to convert it into dDTP. The synthesis involves converting 2-amino-2′-deoxy-adenosine to its 5′-monophosphate derivative dDMP followed by pyrophosphorylation (9,10). Other chemical routes have been reported (11,12). Alternatively, the triphosphate nucleotide dDTP may be produced biotechnologically directly from the base precursor DAP, as is the case with the related nucleobase 2-aminopurine. When added to growing bacteria, 2-aminopurine is metabolized to form deoxy-2-aminopurine triphosphate (dAPTP) (13) which, like dDTP, serves as a substrate for DNA polymerases (14,15). It is known that DAP, which is toxic to cultured cells, is normally metabolized to DAP ribonucleoside and then deaminated to guanosine (16). DAP and its 2′-deoxyriboside (DAPdR) exert their toxicity by different mechanisms. DAPdR, but not DAP, acts as a precursor of deoxyguanosine in mammalian cells (17). The

*To whom correspondence should be addressed. Tel: +33 320 16 92 20; Fax: +33 320 16 92 29; Email: bailly@lille.inserm.fr

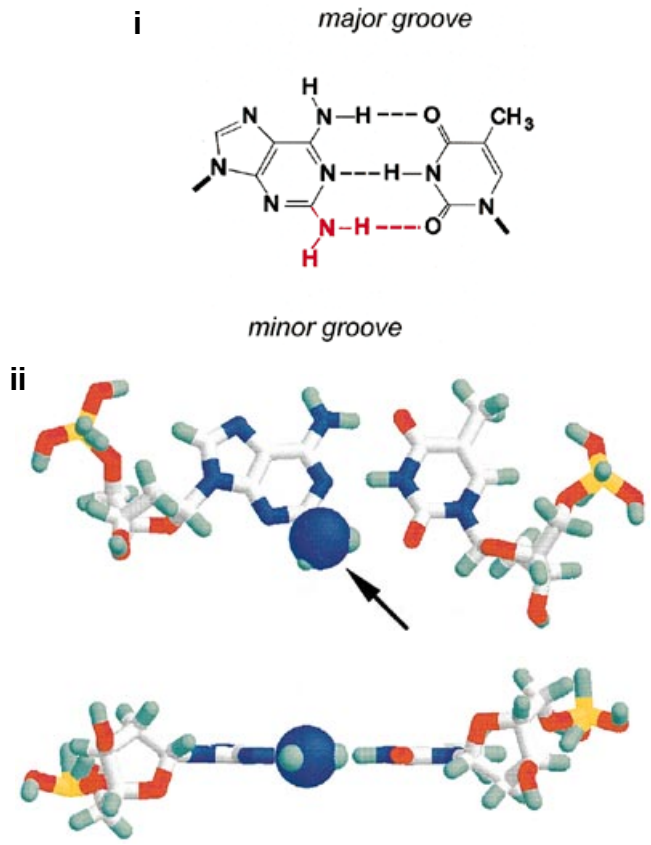

Figure 1. Structure of a diaminopurine-thymidine base pair (Watson–Crick pairing). The positions of the minor and major grooves are indicated. The 2-amino group which distinguishes a D·T pair from an A·T pair is printed in red. Broken lines represent hydrogen bonds. The molecular model was built with the programs HyperChem 5.1 and Alchemy 2000. The guanine 2-amino group is shown as a blue sphere.

polymerization of chemically synthesized DTP by deoxynucleotidyl transferase from calf thymus has been reported (18).

DIAMINOPURINE INCREASES THE THERMAL STABILITIES OF DUPLEX DNA AND RNA

DAP is frequently introduced into nucleic acid sequences to increase the melting temperature of DNA (19) and/or RNA duplexes (20) and for related applications such as primers for sequencing and PCR (21,22) and fingerprinting (23). Incorporation of DAP into short DNA oligomers increases the thermal stability of the duplex by 0–2°C per D·T base pair (24). Prosnyak *et al.* (23) reported that the melting temperature (T_m) of a given oligo or polynucleotide containing *y*% of DAP is increased by a factor of 0.14*y* (ΔT_m). However, dependence of the T_m on the DAP content is not linear (25,26). For a 160 bp DNA fragment having all A replaced with DAP residues on both strands of the fragment, we measured T_m values of 65.9, 77.4 and 78.6°C, whereas the T_m values for the corresponding DNA fragment containing natural bases were 62.7, 70.0 and 72.8°C (27). The T_m elevation resulting from introduction of a 2-amino group onto A residues is much smaller in the deoxy series than in the ribo series (5). In the deoxyribo series the stabilizing contribution arising from the formation of a third hydrogen bond in D·T pairs is opposed by a destabilization due to disruption of the spine of hydration in the minor groove of B-form DNA (28).

The potential uses of DAP are wide ranging. For example, it has recently been used in conjunction with 2-thiothymine to produce selectively binding complementary (SBC) oligonucleotides which can facilitate the formation of particular DNA structure (e.g. three-arm junctions) via strand invasion (29). DAP, like 2,4-diaminopyrimidine, can be used for the development of components of an extended genetic alphabet (30). Very recently, Nielsen and co-workers reported that incorporation of DAP nucleobases into peptide nucleic acids (PNA) increased the DNA binding and sequence discrimination of PNA oligomers (31).

USE OF DIAMINOPURINE BY DNA POLYMERASES

Incorporation of nucleoside triphosphate analogues by polymerases is a method of choice to examine miscoding by different DNA polymerases. For example, 8-oxo-dGTP and 8-amino-dGTP were employed in a recent study comparing the efficiency of utilization of these modified bases by HIV type-1 and murine leukemia virus reverse transcriptases with that of mammalian DNA polymerases (32). The HIV-1 reverse transcriptase readily accepts non-canonical bases, as well as nucleoside triphosphates modified on the sugar (33). Lutz *et al.* (30) have shown that the AIDS virus enzyme successfully incorporates the triphosphate form of 2,4-diaminopyrimidine opposite 2′-deoxy-7-deazaxanthosine, whereas the same reaction failed completely with calf thymus DNA polymerases or with the Klenow fragment of *Escherichia coli* DNA polymerase I. As regards DAP, it is a good substrate for a number of polymerases. Substantial changes in the minor groove of DNA do not disrupt recognition contacts with T3 or T7 RNA polymerases. Apparently, there is no interaction between RNA polymerase and the guanine 2-amino group (34,35). Heat stable polymerases can also function with modified base-containing nucleoside triphosphates. dDTP is readily accepted by *Taq* polymerase and related enzymes. We have shown that incorporation of the diaminopurine nucleobase into both strands of a 160mer fragment presents no particular difficulties (36).

STRUCTURAL STUDIES OF DIAMINOPURINE-CONTAINING DNA

Structural studies of DNA have also profited from the use of DAP. Crystal structures of the hexanucleotides d(CGUDCG)$_2$, d(CGTDCG)$_2$ and d(CDCGTG)$_2$ revealed that substitution with a central D·U or D·T pair is consistent with presumed Z-DNA formation (37,38). The net effect of adding an N2 amino group to the C2 carbon of the adenine base in a T·A base pair is to render the minor groove of both B- and Z-DNA more hydrophilic. Although the effect of the added exocyclic amino group on the stability of a Z conformer is greater than for a B conformer, both the Z and B structures have a C2 carbon that becomes almost entirely inaccessible to solvent upon addition of the N2-amino group (39). Structural studies with DAP-containing oligonucleotides (and other modified bases, in particular inosine) have shown unambiguously that the N2-amino group is critically important in defining the stability of Z- versus B-DNA. It is interesting to note that with the Z-form oligonucleotide d(CDCGTG), just as with the hexanucleotide d(CGCGCG), the continuous spine of water molecules in the minor groove crevice is not disrupted, suggesting that these sets of water molecules help to stabilize Z-DNA conformation (37,39).

The effect of the purine 2-amino group on the preferred conformational states of DNA has also been studied with the synthetic polymer poly(dD-dT)$_2$ (28,40). Spectroscopic analysis of poly(dD-dT) showed that a salt-dependent transition from the standard B-form to an unusual A-form conformation occurs under certain solvent conditions and/or in the presence of polyamines (41–43). The putative A-form of poly(dD-dT) is stabilized by the methyl group at position 5 of the pyrimidine base (44). However, comparative NMR analysis of the dodecanucleotide duplex d(GCATTATTGC) and its analogue having all A replaced by D indicated that the A→DAP substitution does not disturb the global or local conformation of the DNA duplex, at least under low salt conditions (45).

Uranyl nitrate is a sensitive tool for probing the structure of nucleic acids. The extent of photocleavage of double-stranded DNA by uranyl ions at acidic pH (~6.0–6.5) exhibits a very strong modulation which is correlated with minor groove width/electronegative potential (46). The width of the minor groove of the DNA helix is to a first approximation correlated with its AT/GC content since AT-tracts appear to be associated with a narrowed groove, whereas GC-tracts have a widened minor groove. Studies of uranyl-mediated cleavage of DNA containing DAP residues have revealed that the variation of the minor groove width with the local nucleotide composition can be attributed to a large extent to the presence of a purine 2-amino group on G·C base pairs.

As shown in Figure 2, the reactivity towards uranyl nitrate at acid pH is modulated in the DAP-containing DNA quite differently from natural DNA, consistent with a marked widening at sites of A→DAP replacement. The interpretation is clear, that A→DAP substitutions markedly affect the minor groove width of DNA. The changes in groove width are equally evident in the patterns of susceptibility of these two DNA species to DNase I cleavage (47).

The exocyclic amino group of G or D plays a significant role in the intrinsic curvature of DNA. Gel electrophoresis studies employing a series of oligonucleotides 5′-AAAAAGCCGC-3′ where A and G residues were systematically substituted with D or I residues at different positions indicated that the curvature induced by an A-tract in DNA molecules is primarily located at the junction with the 3′-end of the A-tract (48,49).

DIAMINOPURINE AND RNA STRUCTURE AND FUNCTION

The nucleotide analogue interference mapping assay is a sensitive method by which to identify and determine the exact contribution of the chemical groups within RNA which are essential for its activity (50). The technique has recently been used to investigate the role of every N2-exocyclic amine of G within a large RNA, the *Tetrahymena* group I intron, using diaminopurine riboside monophosphate (50). This DAP riboside also proved valuable for studying G·U wobble pairs in a variety of RNA molecules (51,52) and for investigating spliceosome assembly. Antisense probes incorporating DAP are efficiently able to select RNP particles which would otherwise be inaccessible (20). This suggests that DAP may improve antisense activity.

DIAMINOPURINE AND PROTEIN–DNA RECOGNITION

The 2-amino group of guanine residues is the only hydrogen bond donor group exposed in the minor groove of DNA. In addition, it impedes access to the floor of the groove and it interferes with the spine of hydration. As such, it could be expected to play a determinant role in the recognition of DNA sequences by proteins, peptides and small molecules.

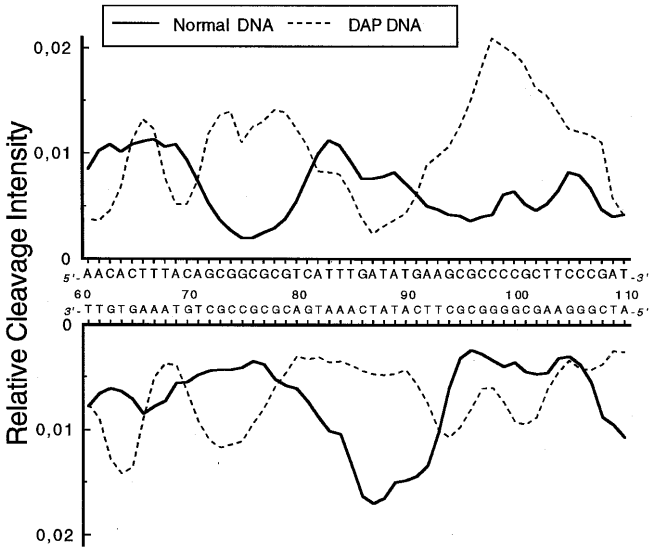

Figure 2. Cleavage plots comparing the susceptibility of the 160 bp *tyr*T DNA fragment containing natural bases or D·T pairs in place of A·T pairs to the uranyl-mediated photocleavage reaction. The double-stranded sequence shown above and below the axis corresponds to that of the normal DNA. In the DAP DNA, all adenine residues are replaced by diaminopurine residues (47).

The crystal structure of the restriction endonuclease *Eco*RI complexed to the tridecamer d(TCGCGAATTCGCG) containing the recognition sequence (underlined) reveals that the enzyme makes contact with the double helix mainly in the major groove (53). Specific hydrogen bonding interactions can be detected between the enzyme and the O6/N7 of guanine and N6/N7 of adenine at the target site. There is no apparent specific interaction involving the N2-amino group of guanine in the minor groove of the duplex at the restriction sequence. As a result, one would anticipate little, if any, perturbation of *Eco*RI cleavage of modified DNA duplexes containing bases with altered 2-amino groups. Nevertheless, various experimental studies have shown that replacement of deoxyguanosines or deoxyadenosines in 5′-GAATTC with deoxyinosines or deoxydiaminopurines, respectively, can significantly change the enzymatic activity of *Eco*RI (54,55). For example, substitution with DAP at position 3, d(GADTTC), resulted in a 9-fold decrease in the specificity constant (54). The use of DAP-containing substrates has provided valuable clues to the mechanism by which *Eco*RI and related enzymes (e.g. *Rsr*I; 56) recognize the duplex sequence GAATTC. Similar results have been reported with other restriction endonucleases that mainly bind and cut via the major groove of DNA (10,57). DAP has also been used to study the kinetics of DNA methylation by the *Eco*RI modification methylase and *Dam* methyltransferase (58,59).

Thus, although *Eco*RI can be considered essentially as a major groove-binding protein, the nuclease is evidently also sensitive to the functional groups exposed in the opposite minor groove. The same conclusion was reached when we studied the interaction between the factor for inversion stimulation (FIS) and DNA containing inosine and/or DAP residues. FIS is a major groove-binding protein from *E.coli* required for several processes, including site-specific recombination, transcriptional activation

and DNA replication (60). We have shown that base substitutions which alter the placement and presence of the purine 2-amino group in the minor groove can affect both the intrinsic curvature and the bendability of DNA and thereby modulate the interaction with proteins like FIS, whose binding sites lie purely within the major groove of the double helix (61). Two other proteins whose interaction with DNA is strongly affected by A→D and G→I replacements are HMG-D, a member of the HMG-1 family of chromosomal proteins (62), and the integration host factor (IHF) from *E.coli*, a small protein which binds preferentially to AT-rich sequences. Binding studies with a series of 41 bp oligonucleotides containing adenine analogues revealed that the interaction of IHF with the double helix involves contacts within both the minor and major groove. A→D substitution within the binding region considerably reduces the protein binding affinity (up to 50-fold) (63). For both HMG-D and IHF, the effect is attributable to an indirect influence mediated via helix deformability.

DIAMINOPURINE AND DNA REPAIR

Owing to the structural similarities between D·T and G·T pairs, DAP has been used to study the repair of G·T mismatches. Experiments using cell-free extracts and 45 bp oligonucleotides containing D·T pairs at defined positions suggest that the repair mechanism operating on D·T pairs may be the same as the human G·T repair pathway (64). However, more recent *in vitro* studies have revealed marked differences as regards the extent of incision by human thymine glycosylase of 45 bp heteroduplexes bearing G·T mispairs or D·T pairs (65).

DRUG–DNA RECOGNITION STUDIES USING DIAMINOPURINE

About 30 years ago the idea was first proposed that the 2-amino group of guanine is a significant determinant for the binding of small molecules within the minor groove of the double helix. Cerami *et al.* (66) studied binding of the antitumour drug actinomycin (an antibiotic that remains extensively used in cancer chemotherapy) to poly(dI)·(dC) and to a synthetic analogue of poly(dA-dT)·(dA-dT) containing DAP bases partly or wholly replacing the adenine bases. This was the first experiment using polydeoxynucleotides in which the purine 2-amino group was deleted (G→I substitution) or added to adenine residues (A→D substitution). Later, short oligonucleotides containing DAP residues were used as substrates for DNA binding/cleavage studies. Using hexanucleotides possessing A·T, G·C, I·C or D·T pairs, Sugiyama *et al.* (67) investigated the mechanism of DNA cleavage by the antibiotic neocarzinostatin. Gao *et al.* (68) also used a hexanucleotide, d(CGTDCG)$_2$, to refine the X-ray structure of the covalent formaldehyde-mediated complex between this DAP-containing duplex and the daunorubicin derivative MAR-70.

Since the pioneer work of Cerami *et al.* (66), the influence of the 2-amino group of guanine on drug–DNA recognition has always been considered critical, but its exact role has remained uncertain. It is only recently that its precise function has been elucidated, through the combined use of PCR technology and base-modified nucleoside triphosphates, which enabled us to determine how and to what extent the 2-amino group of guanine contributes to the recognition of specific DNA sequences by a large diversity of small molecules.

Sequence-specific cleavage of DNA by the antitumour antibiotics bleomycin and calicheamicin γ_1^I is strongly dependent on the position of the purine 2-amino group. For bleomycin, relocating the 2-amino group from guanine to adenine nucleotides creates new cleavage sites at pyrimidine residues lying 3′ of DAP residues. For calicheamicin, the presence of a purine 2-amino group adjacent to the cutting site potentiates the cleavage reaction (69). The 2-amino group also constitutes a key structural element for sequence-specific recognition of DNA by non-covalent binders. Irrespective of their mode of interaction with the double helix, such GC-specific antibiotics as mithramycin and chromomycin find new binding sites associated with DAP-containing sequences in (I+DAP)-substituted DNA and are excluded from former canonical sites containing I·C base pairs. The converse was found to be the case for a group of normally AT-selective ligands which bind in the minor groove of the helix, such as netropsin, berenil and DAPI (36,62,70). The binding sites of almost all DNA-binding drugs and antibiotics strictly follow the placement of the purine 2-amino group, which serves as both a positive and negative effector (69).

To illustrate the effect of DAP residues on drug–DNA recognition, a footprinting gel obtained with the antitumour antibiotic mithramycin is presented in Figure 3. The effect of shifting the purine 2-amino group from guanines to adenines (by virtue of combined A→D and G→I substitutions) is to provoke a significant redistribution of binding sites for mithramycin, such that the newly created sites containing D·T pairs are substantially preferred over G·C-containing sites (36). For example, the strong footprint around position 100 with normal DNA in the presence of mithramycin is totally absent with the modified DNA species and, conversely, the footprint around position 87 with I+DAP DNA corresponds to a region of enhanced DNase I cleavage with normal DNA. Also, the strong footprint observed around position 75 with normal DNA is missing in DNA containing both I and DAP residues. There is no doubt that the pattern of binding sites for mithramycin is radically changed. The drug is displaced from its G·C sites in natural DNA to pick up new sites in the DAP·T-rich sequences created in the modified nucleic acid.

The most pronounced effects attributable to DAP were observed with the quinoxaline antibiotics such as echinomycin and triostin A (71,72). The A→D substitution potentiates enormously the interaction of these two drugs with DNA, up to 1000-fold. With normal DNA, a concentration in the 10–20 μM range is needed to evidence strong binding to CpG sites. With the DAP-containing DNA, footprints are already very pronounced at only 0.5 μM drug and binding to newly created TpD sites can be unambiguously detected at a concentration as low as 10–20 nM (72). This enhancement of binding is only seen with the naturally occurring quinoxaline antibiotics and does not occur with the synthetic analogue TANDEM, which recognizes A·T-containing sites and is totally insensitive the relocation of the exocyclic amino group (72). In this case, the binding specificity may arise primarily from stacking and hydrophobic interactions rather than from direct contact with the exocyclic guanine 2-amino group (72). With other drugs, such as actinomycin, the extent of binding to DAP·T sites is essentially unchanged compared with normal DNA (71). There is something special about regions of alternating T·D base pairs which generates unusually good binding sites for echinomycin and triostin A. The local structure and/or the rigidity of the TpD sites could be exploited by the drugs to fit particularly neatly within the minor groove. Alternatively, the stacking of their quinoxaline rings upon DAP·T base pairs could be especially propitious (73).

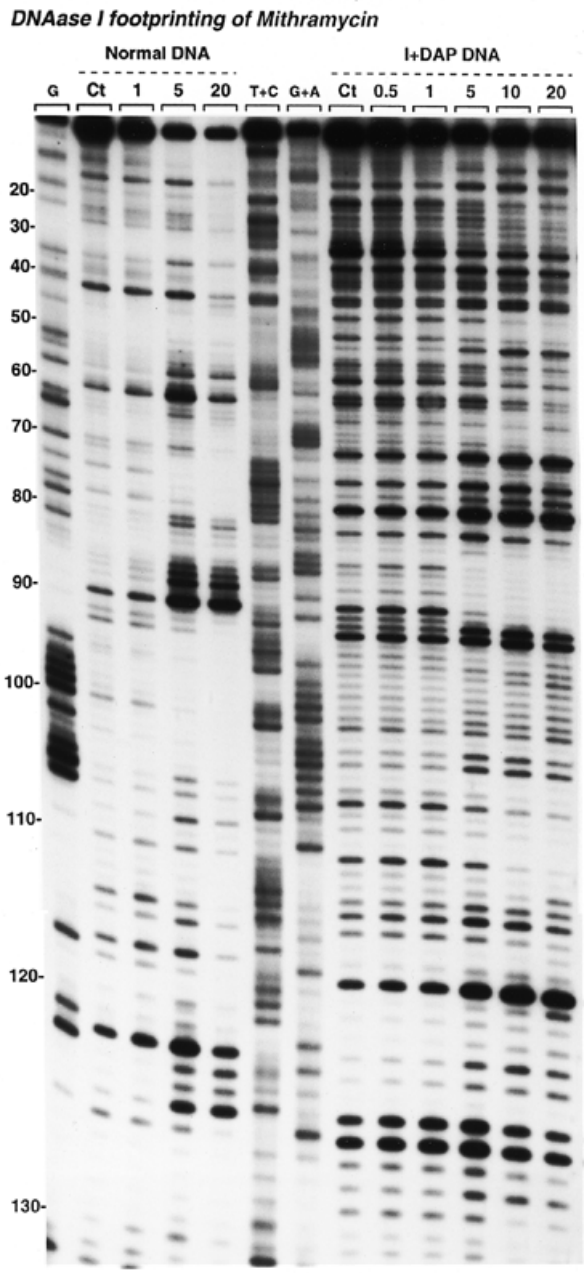

Figure 3. DNase I footprinting of mithramycin on the Crick (sense) strand of normal and inosine + DAP-containing *tyr*T(A93) DNA. The products of DNase I digestion were identified by reference to the Maxam–Gilbert markers (lanes T+C and G+A). Control lanes (Ct) show the products resulting from limited DNase I digestion in the absence of drug. The remaining lanes show the products of digestion in the presence of the indicated antibiotic concentrations (expressed as micromolar). The scale on the left corresponds to the standard numbering of the *tyr*T DNA as represented in Figure 2.

There are very few cases where the replacement of adenine with DAP has little or no effect on the sequence selectivity associated with drug binding. The footprinting profile of the 'threading' intercalator nogalamycin is potentiated in DAP+I-substituted DNA but otherwise remains much the same as with natural DNA (36). Studies with a series of antitumour bis-naphthalimide derivatives which bis-intercalate into DNA sequences, particularly those containing GpT (ApC) and TpG (CpA) steps, showed that repositioning the 2-amino group of G·C base pairs by substitution with inosine and/or DAP had little effect on the distribution of drug molecules between binding sites. In contrast to nearly all common (bis)intercalating drugs, the bis-naphthalimides appear to engage in contacts with the edges of the base pairs via the major groove of the double helix (74).

CONCLUSION

After incorporation of DAP into DNA, the significant difference from adenine is that a 2-amino group is present in the minor groove (Fig. 1). The consequent modification of the surface of the minor groove results in altered conformational properties of the double helix, leading to altered recognition by proteins and small molecules. The D·T base pair is more stable than an A·T pair. The reinforcement of base pairing reduces the flexibility of the DNA and thus generally reduces the extent of protein binding, at least for small proteins such as FIS, HMG-D and IHF. A→D substitutions also decrease the capacity for binding within the minor groove of antibiotics such as netropsin and distamycin, whereas for other drugs, like the quinoxaline antibiotics echinomycin and triostin, the DAP substitution promotes the recognition process considerably. It is likely that the larger D·T pair (having a dipole moment of ~2.3) gives rise to better stacking interactions with an intercalating chromophore than the standard A·T pair (dipole moment ~1.7) (75–77). The changes in surface area and electrostatic properties of the base pair may favour interaction with a planar chromophore but the strength of intercalation must also depend on the interaction of the attached groups (e.g. the peptide moiety of echinomycin or the sugar moiety of calicheamicin) with the sequences flanking the intercalating site. The approach described here, which uses modified bases to study ligand–nucleic acid interactions, provides useful information, but one should bear in mind that the observed effects can arise from direct interaction between the ligand and the newly introduced group on the base, as well as from indirect interactions. Analogue substitution, be it with DAP or another base, can always affect the position or properties of neighbouring bases that might be involved in direct interactions with the ligand.

In addition to the varied applications presented above, DAP has been used for the recognition of abasic sites in DNA (78), to investigate mechanisms of mutagenesis (79,80) and to select purine-resistant variants from mutagenized cultures of *Drosophila* (81). Thus, the potential applications of DAP as a nucleobase cover many modern aspects of nucleic acid chemistry from structure to biology. DAP represents a useful chemical product particularly well suited to increase the stability of double-stranded nucleic acids. At the same time it is a natural product and a key element of the genetic machinery of the S-2L cyanophage. The ability of polymerases to accept non-standard base pairs, such as D·T pairs, is remarkable in the light of the physiological role that the polymerases play. Studies with DAP and other modified bases reinforce the idea that the Watson–Crick formalism can be extended while maintaining a high fidelity of DNA replication which is essential for preserving the integrity of living organisms. DAP-containing nucleic acids can be prepared in many synthetically convenient ways so as to produce different repertoires of molecules having a range of functionalities. There is good reason to believe that the successful use of DAP will continue to inspire the development of novel nucleic acid products and the emergence of new techniques.

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